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Transient depletion of xDnmt1 leads to premature gene activation in *Xenopus* embryos

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In *Xenopus* laevis zygotic transcription begins at the midblastula transition (MBT). Prior to this the genome is organized into chromatin that facilitates rapid cycles of DNA replication but not transcription. Here we demonstrate that DNA methylation contributes to the overall transcriptional silencing before MBT. Transient depletion of the maternal DNA methyltransferase (*xDnmt1*) by anti sense RNA during cleavage stages is associated with a decrease in the genomic 5-methyl-cytosine content and leads to the activation of zygotic transcription approximately two cell cycles earlier than normal. Hypomethylation allows the early expression of mesodermal marker genes such as *Xbra*, *Cerberus*, and *Otx2*, which are subsequently down-regulated during gastrulation of the *xDnmt1*-depleted embryos. The temporal switch in gene expression may account for the appearance of body plan defects that we observe. Loss of *xDnmt1* can be rescued by the coinjection of mouse or human Dnmt1 protein. These results demonstrate that DNA methylation has a role in the regulation of immediately early genes in *Xenopus* at MBT.

[*Key Words*: 5-methylcytosine; *Xenopus*; DNA methyltransferase; antisense RNA; MBT]

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The idea of a heritable, but alterable, switch on DNA has an obvious appeal as it could allow for the regulation of a repertoire of genes during embryo development. One candidate for this role as a global modifier of gene activity in vertebrates is DNA methylation, the pattern of which can be inherited in a relatively stable form in somatic cells (Colot and Rossignol 1999). DNA methylation near promoters or enhancer elements leads to the stable inactivation of the associated gene in vitro and in vivo (Bird 1992; Kass et al. 1997a,b). Methylated CpG pairs act as specific ligands for transcriptional repressors (MeCP1 and MeCP2) leading to stable gene inactivation (Jones et al. 1998; Nan et al. 1998). Methyl groups are introduced onto cytosine by enzymes known as DNA methyltransferases (Dnmt), which have been cloned and characterized in many species (Colot and Rossignol 1999). At least three paralogs of this family exist in mouse (Okano et al. 1998) and the most abundant and metabolically active protein, Dnmt1p, was originally characterized as a maintenance methyltransferase (Bestor and Ingram 1983). Hemimethylated DNA is the favored substrate for Dnmt1p, but it also has significant de novo methyltransferase activity in vitro (Pradhan et al. 1997).

DNA methylation is necessary for normal mouse development as embryos homozygous for a loss-of-function mutation (*Dnmt1*$^{n/n}$) in the DNA methyltransferase gene die in midgestation (Li et al. 1992), showing evidence of developmental delay and aberrant expression of imprinted genes (Li et al. 1992, 1993). Complex changes in DNA methylation patterns occur during mouse development and cell differentiation that involve a genome-wide demethylation during cleavage followed by a wave of de novo methylation in the growing embryo (Monk et al. 1987; Razin and Kafri 1994). These changes have often been correlated with hypomethylation at tissue-specific loci in the different somatic lineages. However, this view has been challenged by a recent study that showed that many tissue-specific gene promoters are not methylated or expressed in early embryos (Walsh and Bestor 1999). In the case of mammals, it is argued that DNA methylation only has a role in specialized processes such as the maintenance of X-inactivation or imprinting in somatic cells. An examination of *Dnmt1*$^{n/n}$ mouse embryos has not clarified this point because the observed phenotypes are complex (Li et al. 1992; Lei et al. 1996; Trasler et al. 1996).

Xenopus laevis has a CpG methylation system similar to that of mammals, but relatively little is known about the dynamics of DNA methylation during development. There is no evidence for a global demethylation, imprinting, or inactivation of sex-specific chromosomes in *Xenopus* (Thiebaud et al. 1984; Tymowska 1991; Ya-

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mada et al. 1999). The low efficiency of nuclear transplantation experiments (Gurdon et al. 1975), very similar to that in mammals, suggests that epigenetic mechanisms may have a role in determining nuclear competence during *Xenopus* development.

A period of global transcriptional silence is observed between formation of the activated egg and the midblastula in *Xenopus.* There are probably several separable events that regulate this developmental switch (known as the midblastula transition or MBT) when there is as much as a 50-fold increase in the transcription of some genes after the 12th cleavage division (Newport and Kirschner 1982a,b). Experimental evidence suggests that initially chromatin assembly, facilitated by the large pool of maternal histones, is dominant over the construction of the basal transcription complex (Prioleau et al. 1994; Almouzni and Wolffe 1995) and prevents gene activation. Approaching MBT, the competition at promoters can be reversed in favor of the transcription complex when the maternal histone store is lowered and the replication of DNA becomes coupled with histone synthesis. We are interested in whether DNA methylation can contribute to gene silencing before the MBT in *Xenopus* embryos. An *X. laevis* oocyte form of DNA methyltransferase (*xDnmt1*) has been cloned and the identified protein is highly homologous to Dnmt1 proteins from other vertebrates (Kimura et al. 1996). However, the expression pattern of the *xDnmt1* gene and its requirement during embryogenesis have not been established. We cloned a partial cDNA (1.4 kb) corresponding to the conserved methyltransferase catalytic domain and used it as a probe to follow the expression of *xDnmt1* throughout development. Double-stranded RNA hybrids (caused by antisense RNA injection) in *Xenopus* embryos are eliminated by endogenous nuclease activity leading to the loss of the endogenous mRNA and its associated protein (Lombardo and Slack 1997; Steinbeisser et al. 1995). Our results show that antisense RNA depletes the maternal *xDnmt1* but not the zygotic form of the enzyme, leads to hypomethylation of the genome during the first embryonic cleavages, allows the inappropriate activation of developmentally decisive genes, and affects the early events of cell differentiation at the onset of gastrulation.

Results

Expression of xDnmt1 *during* Xenopus *development*

We isolated a 1.4-kb somatic *xDnmt1* clone, *xDnmt1*p9/19, by screening a stage 20–22 cDNA library (Fig. 1A). The clone had an open reading frame of 373 amino acids and showed 98% identity with the carboxy-terminal catalytic domain of the full-length (1490 amino acids) oocyte form of *xDnmt1* (Kimura et al. 1996). The protein motifs VIII, IX, and X that are essential for enzyme activity are identical between the somatic and oocyte forms of *xDnmt1* (data not shown).

Northern blot analysis with a *xDnmt1*p9/19 probe shows that *xDnmt1* or related transcripts are present throughout development (Fig. 1B, top). An mRNA of 5 kb is observed as an abundant maternal transcript in the mature oocyte and egg. After midblastula (stage 8.5) and during gastrulation (stage 12) the maternal *xDnmt1* mRNA is replaced by a somatic form that is present at low levels between stages 16 and 23 and increases at stage 36 (late tadpole). The relative changes in *xDnmt1* mRNA levels during development are compared with that of ubiquitously expressed ornithine decarboxylase gene (*ODC*) in Figure 1B.

For a more detailed analysis of *xDnmt1* expression patterns, whole-mount in situ hybridization was performed on eggs and embryos from various stages. The majority of the maternal *xDnmt1* transcript localizes to the animal pole in the egg and early blastula but is hardly detectable in the vegetal pole (Fig. 1C–E). To test whether the differential localization of *xDnmt1* transcripts in cleavage stage embryos is not an in situ hybridization artifact and reflects the distribution of methyltransferase protein in the early embryo, *xDnmt1*p was immunoprecipitated from extracts derived from dissected animal and vegetal halves of the 64-cell blastulae. Western analysis with a monoclonal anti-Dnmt1p antibody (see Materials and Methods for details) of the immunoprecipitated material reveals that xDnmt1p is very abundant in the extract of animal hemisphere cells and virtually undetectable in the vegetal counterpart (Fig. 1K, top). In contrast proliferating cell nuclear antigen (PCNA) is present in equal amounts in both the animal and the vegetal cell extracts (Fig. 1K, bottom). The zygotic form of the *xDnmt1* transcript in stage 12.5 gastrulae can be observed in the deep cells of the dorsal and ventral mesoderm (Fig. 1F) and in the presumptive neural ectoderm during neurulation (Fig. 1G). Generally, a similar pattern of expression is maintained during the tailbud and tadpole stages (Fig. 1H–J). Comparable whole-mount in situ studies in mouse and zebrafish have shown a very similar localization of Dnmt1 and persistently high levels of methyltransferase in neural lineage cells (Goto et al. 1994; Martin et al. 1999). It is conceivable that *xDnmt1* is actually expressed in all cells at varying levels, but this will require a more detailed analysis of individual tissues.

Depletion of maternally expressed xDnmt1

To determine whether the maternal methyltransferase is essential for early development, animal or vegetal regions of the early embryo were injected with sense or antisense RNA derived from the p*xDnmt1*-9/19 cDNA clone. Control and injected embryos were cultured until the equivalent of the tadpole stage 35 and monitored during this time for their phenotypic appearance. Alternatively, they were collected at early to midblastula and gastrula stages and analysed for the presence of *xDnmt1* RNA and protein.

Figure 2A summarizes the results of the microinjection experiments. Animal blastomeres were targeted first as this is where we observed the highest levels of *xDnmt1* mRNA. The injection of 520 pg per cell of sense RNA into the animal pole of two- and four-cell blastulae

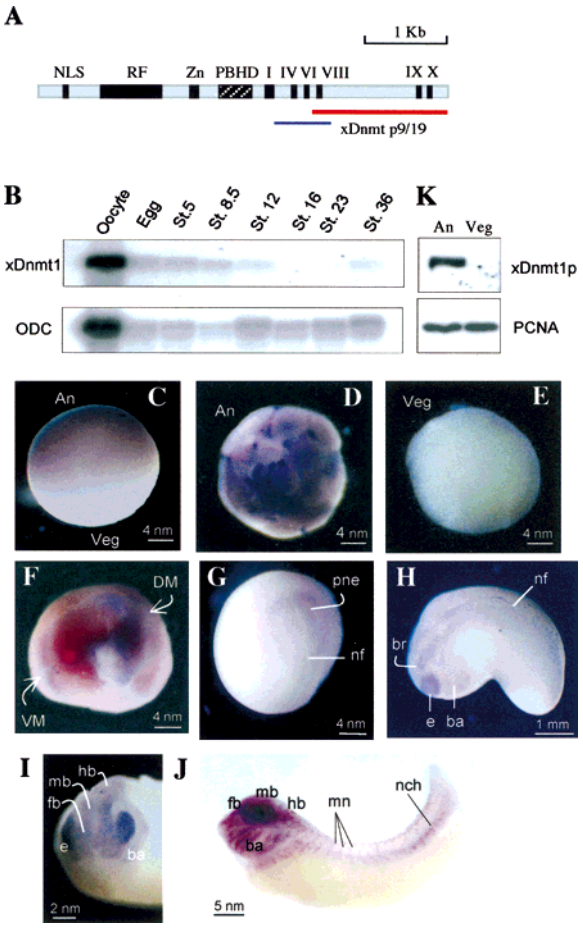

Figure 1. Expression of *xDnmt1* during *Xenopus* development. (*A*) The functional domains of the xDnmt1 protein are shown as shaded and solid boxes. (NLS) Nuclear localization signal; (RF) region involved in targeting of the enzyme to replication foci; (Zn) cystine-rich Zn/DNA-binding motif; (PBHD) polybromo-1 protein homologous domain; I–X are the conserved motifs of the methyltransferase catalytic domain. The 1.4-kb partial somatic *xDnmt1* cDNA (st. 20–22) is 98% homologous to the oocyte *xDnmt1* catalytic domain (red). The blue bar indicates the PCR product used to detect *xDnmt1* depletion in Figure 3A. (*B*) Northern blot analysis of *xDnmt1* transcripts during embryo development. Ornithine decarboxylase (ODC) is an ubiquitously expressed gene. Stages of development are indicated above the *xDnmt1* blot. (*C*,*D*) Whole-mount in situ hybridization localizes *xDnmt1* transcripts (pink) to the animal pole of albino *Xenopus* egg (*C*) and animal pole blastomeres of 64-cell blastula (*D*). (An) Animal pole; (Veg) vegetal pole. (*E*) *xDnmt1* is not detected in the vegetal hemisphere of 64-cell blastula. (*F*) After MBT the somatic form of *xDnmt1* appears in the deep cells of the dorsal (DM) and ventral (VM) mesoderm of stage 11 gastrula (sagittal section). (*G*) During neurulation at stage 15 the staining for *xDnmt1* transcripts is localized in the eye (e) and brain regions (br) of the prospective head neuroectoderm (pne) and along the edges of the open neural fold (nf). (*H*,*I*) A similar pattern is maintained during tailbud stages 20 (*F*) and 23 (*G*). (fb) Forebrain; (mb) midbrain; (hb) hindbrain. (*J*) At stage 35 (tadpole) the methyltransferase probe stains eyes, brain regions, branchial arches (ba), posterior notochord (nch), and the motor neurons (mn). (*K*) xDnmt1 protein (xDnmt1p) was immunoprecipitated from extracts derived from animal or vegetal halves of 64-cell blastulae and detected by Western blot analysis. Both extracts contain equal amounts of PCNA (bottom).

had no noticeable effect on embryo survival or appearance when compared with noninjected controls (Fig. 2D,E). In contrast significant developmental abnormalities appear when antisense *xDnmt1* RNA is injected into two- and four-cell embryos at a dose of >120 pg per cell. Embryos injected with 520 pg of antisense exhibit considerable delay in closing the blastopore in comparison with control embryos of the same stage (Fig. 2B,C). When they were allowed to develop further, doses of 400 and 520 pg of antisense led to the appearance of a microcephalic phenotype (Fig. 2F) in some cases accompanied by axis truncation (Fig. 3F). A considerable number of the antisense-injected embryos die during gastrulation and neurulation (Fig. 2A). An even more severe effect was caused by injection of 400 pg of antisense RNA into each of the animal blastomeres of 8- and 16-cell stage where 90% of the embryos at stage 35 either exhibit severe developmental defects or arrest at gastrulation and neurula stages. To test whether the effect of the antisense was specific to animal blastomeres, 520 pg of this RNA was injected into the each vegetal cell of four- and eight-cell blastulae. The vast majority of these developed normally.

To test whether the injection of *xDnmt1* antisense RNA leads to loss of the endogenous maternal transcript we performed quantitative RT-PCR reactions with a pair of primers overlapping the junction of sense and antisense RNA (see Fig. 1A). Fig. 3A shows that 600 pg of antisense RNA per cell injected into each animal pole blastomere of 2- and 4-cell embryos is sufficient to decrease the level of the maternal *xDnmt1* transcript to <5% of that detected in the normal 64-cell blastula. *xDnmt1* protein is also barely detectable in the depleted blastula stage embryos (Fig. 3B). At gastrula, to our surprise, both *xDnmt1* mRNA and protein are upregulated in the antisense injected embryos (Fig. 3A,B). These results were also confirmed by in situ hybridization with a 5′ *xDnmt1* probe (Fig. 3C,D). The unstable antisense RNA does not appear to interfere with the zygotically expressed form of *xDnmt1*, as the *xDnmt1* transcript and protein appear at much higher levels in the antisense injected gastrulae (see Fig. 3A,B) compared with the normal embryos. It is clear that a transient depletion of *xDnmt1* during blastula stages causes developmental abnormalities and sets in motion a series of events that results in subsequent changes in the pattern of expression of the zygotic form of *xDnmt1*.

Rescue of the antisense xDnmt1 *RNA phenotype*

To ascertain whether the phenotype of *xDnmt1* depleted embryos is due to the loss of methyltransferase activity we attempted to rescue the antisense embryos by coinjection of antisense *xDnmt1* RNA with either human or mouse Dnmt1 proteins (Pradhan et al. 1997) into the animal hemisphere of two- and four-cell blastulae. Coinjection with hDnmt1p or mDnmt1p reverses the antisense RNA effect in a dose-dependant manner (Fig. 4A). hDnmt1p (4 pg) cannot change the mutant phenotype of the antisense injected embryos (Fig. 4B), whereas 12 pg of

A

Phenotypes of xDnmt 9/19 RNA injected embryos

xDnmt 9/19 RNA microinjected	Total n	Normal n (%)	Microcephaly n (%)	Microcephaly, truncated axis n (%)	Die at gastrula n (%)	Die during neurulation n (%)
-	60	56 (93.3)	1 (1.6)	0	3 (5)	0
2 and 4 cell **520** pg **sense**	60	54 (90)	3 (5)	0	3 (5)	0
2 and 4 cell **An. 120** pg **antisense**	79	63 (79.7)	7 (8.8)	0	5 (6.5)	4 (5.2)
2 and 4 cell **An. 400** pg **antisense**	39	18 (46.2)	5 (12.5)	6 (15.4)	10 (25.6)	6 (15.3)
8 and 16 cell **An. 400** pg **antisense**	42	4 (9.5)	10 (23.8)	13 (30.9)	7 (16.6)	8 (19)
2 and 4 cell **An. 520** pg **antisense**	82	27 (32)	14 (7.7)	26 (29.2)	11 (13.4)	6 (7.3)
4 and 8 cell **Veg. 400** pg **antisense**	56	46 (88.5)	2 (3.8)	0	2 (3.8)	2 (3.8)

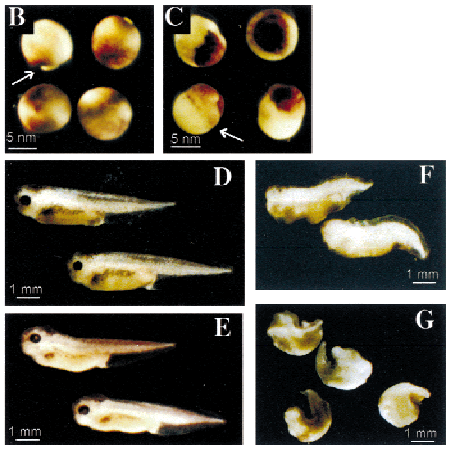

Figure 2. Phenotypes of sense and antisense *xDnmt1* RNA-injected embryos. (*A*) Table showing the phenotypes of stage 35 embryos after injection of synthetic *xDnmt1* RNA. (*B*) Four control embryos (stage 12.5–13) undergoing normal gastrulation and almost closing the blastopore (indicated by arrow). (*C*) The injection of 520 pg of antisense *xDnmt1* RNA into the animal pole at two-cell stage slows gastrulation and disturbs the convergent extension movements of ectomesoderm. Shown are four representative gastrulae at the equivalent of stage 12.5–13. Note the extended appearance of the embryos and the large blastopore (indicated by arrow). (*D*) Uninjected control embryos at stage 35. (*E*) Injection of 520 pg of sense *xDnmt1* RNA into two- and four-cell blastulae or 400 pg of antisense RNA into the vegetal pole does not lead to any visible developmental abnormalities. (*F*) The injection of a low dose antisense RNA (400 pg) into the animal pole of two- and four- cell blastulae causes the embryos to develop microcephally as shown at the equivalent of stage 35. (*G*) The injection of a high concentration (520–600 pg) of the antisense RNA leads to considerable shortening of the dorsal axis in addition to microcephally.

hDnmt1 or mDnmt1 can restore their appearance in 85–90% of the cases (Fig. 4C). In the remaining cases (5–10%) the rescue is only partial where the embryos have normal heads but still exhibit axis defects (Fig. 4D). This suggests that either cross-species methyltransferase protein cannot completely substitute for the function of *xDnmt* or, alternatively, that the endogenous concentration and distribution of xDnmt1p cannot be properly mimicked by microinjection of recombinant proteins. Higher amounts of hDnmt1 (40 pg of protein per embryo) coinjected with the antisense *xDnmt1* RNA result in embryos that develop posterior defects very similar to these caused by an injection of 12 pg of hDnmt1 protein alone into the cells of the vegetal hemisphere (Fig. 4F). When injected into animal pole cells, 12 pg of hDnmt1 protein causes a "no-axis" phenotype (Fig. 4E) and there is a failure to develop past gastrulation. The injection of a comparable amount of BSA had no effect on development in normal or mutant embryos. These experiments imply that, to a large extent, xDnmt1p function can be substituted by cross-species DNA methyltransferases during *Xenopus* embryo development. In addition higher than normal levels of *Dnmt1* proteins cannot be tolerated either by the animal or vegetal cells of the embryo.

DNA methylation levels in normal and antisense xDnmt1*-injected embryos*

We wished to know whether loss of the *xDnmt1* transcripts leads to changes in DNA methylation levels during the cleavages of the antisense injected depleted embryos. A 5-methyl-cytosine (m^5C) monoclonal antibody (Reynaud et al. 1992; Tweedie et al. 1997) was used as a probe for the methylation content in DNA isolated from various stages of normal and antisense RNA-injected embryos along with control DNA samples from *Xenopus* blood, sperm, and the yeast, *Saccaromyces cerevisiae*. As expected, the antibody detects *Xenopus* blood and sperm DNA samples from the normal embryos but the signals progressively decreases towards gastrulation (64 cell and stage 7) and is restored to the initial levels at late neurala stages. We did not observe any global demethylation and remethylation of DNA during these particular stages (Fig. 5A, C, WT); if such an event takes place then it may occur prior to the 64 to cell stage or alternatively in a specific subset of cells in the developing embryo. The latter possibility is supported by our observation that DNA derived from the animal pole cells is up to three times more methylated than the equivalent sample of vegetal pole from 64- to 128-cell blastulae (Fig. 5A,C), which correlates with the polarized localization of *xDnmt1* transcripts and protein in the cleavage stage embryos (see Fig. 1D,K). More detailed analysis will be required to clarify the significance of these differences in methylation levels between distinct subsets of cells in the pre-MBT *Xenopus* embryo. In contrast to the controls, the antibody was almost unable to detect the sample from stage 7 antisense injected embryos DNA although it could detect the 64-cell stage and stage 12 samples (Fig. 5A,C, AS). Later stages (19–35) gave a signal that was comparable to and even slightly higher than that of the control embryo samples. The blot was stripped and reprobed with a mixture of total *Xenopus* and yeast DNA to illustrate the equal loading of DNA on

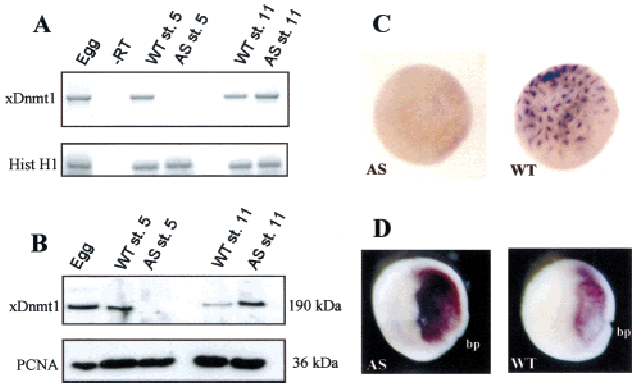

Figure 3. Microinjection of antisense *xDnmt1* RNA transiently depletes the maternal *xDnmt1* transcripts and protein during cleavage stages. (*A*) RT-PCR analysis of *xDnmt1* in RNA samples from wild-type blastulae (WT st.5) and antisense RNA-injected blastulae (AS st.5), wild-type (WT st.11) gastrulae, and antisense-injected (AS st. 11) gastrulae. The location of RT-PCR product with respect to *xDnmt1* sequences and the antisense RNA is shown in Fig. 1A. Histone H1 (Hist H1) transcripts are present in equal amount in all RNA samples. (*B*) Protein extracts derived from wild-type (WT) and antisense RNA-injected (AS) embryos as in *A* were analyzed for the presense of *xDnmt1* by a carboxy-terminal polyclonal antibody. PCNA immunodetection is a loading control. (*C*) In situ hybridization with a 5′ *xDnmt1* probe confirms that the *xDnmt1* transcripts (purple) are missing from the antisense-injected stage 6 blastula (AS) in comparison to a control embryo (WT) at the equivalent stage (viewed from the animal pole). (*D*) Sagittal section after in situ staining (magenta) with 5′ *xDnmt1* probe of stage 10 embryos shows that the zygotic *xDnmt1* transcript is overproduced during gastrulation in the maternal *xDnmt1*-depleted embryo (AS) in the same region as in the control gastrula (WT). Dorsal is to the right; (bp) blastopores.

the filter (Fig. 5B). We obtained similar results using methylation-sensitive restriction enzymes (data not shown). The observed changes in m^5C content appear to reflect changes in *xDnmt1* mRNA and protein levels in both normal and *xDnmt1*-depleted embryos.

Depletion of xDnmt1 leads to premature activation of transcription at MBT

Because DNA methylation is known to repress transcription via the binding of transcriptional repressors, we asked whether the abnormalities in development that we see might be due to the premature gene activation through a global hypomethylation. Zygotic transcription usually initiates at stage 8.5 (also known as MBT) of development. As a test of transcriptional activation, we measured the incorporation of α-^{35}S-labeled UTP in control, sense, and antisense *xDnmt1* RNA-injected blastulae and gastrulae (Fig. 6A,B). In each case RNA was prepared from three sets of staged embryos (15 embryos per time point) that had been microinjected with α-^{35}S-labeled UTP. The control and sense-injected embryos did not incorporate label above background levels until stage 9 of development (Fig. 6A). In contrast we detected up to a fourfold increase in incorporation of [α-^{35}S]UTP much earlier in the *xDnmt1*-depleted embryos at stage 7, but the level of activation is <50% of that seen at stage 9 for control, sense-, and antisense-injected embryos (Fig. 6, cf. A and B). This experiment led us to conclude that *xDnmt1*-depleted embryos initiate zygotic transcription approximately two cell cycles before MBT. To determine which type of genes, either transcribed by RNA polymerase I, II, or III, contribute to the increased incorporation of [α-^{35}S]UTP in antisense-injected blastulae we made use of the differential sensitivity of RNA polymerases to the inhibitor, α-amanatin. Low concentrations of α-amanatin (0.2 µg/ml) inhibit RNA polymerase II transcription only, and this led to a 50–60% decrease in

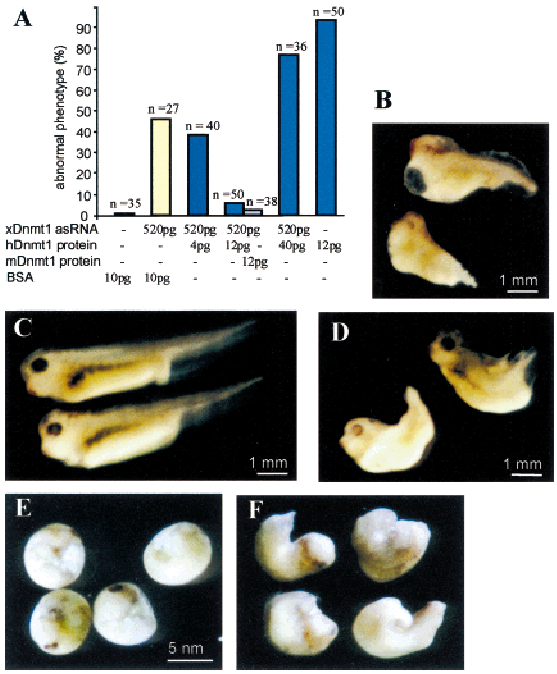

Figure 4. Cross-species *Dnmt1* proteins can substitute for the function of maternal *xDnmt1* depleted by antisense RNA injection. (*A*) Microcephalic and axis-truncated phenotype of *xDnmt1*-depleted embryos can be rescued in a dose-dependent manner by coinjection of recombinant human (hDnmt1) or mouse (mDnmt1) Dnmt1 proteins with 520 pg of antisense *xDnmt1* RNA. The percentage of resulting abnormal embryos scored at stage 35 was plotted for the experiments indicated underneath the bars. (n) Number of injected two-cell blastulae. Yellow indicates injection of antisense only; blue indicates injection of antisense plus recombinant hDnmt1p protein; light blue indicates injection of antisense plus recombinant mDnmt1p protein. (*B*) Both proteins (4 pg) cannot rescue the microcephalic phenotype. (*C*) Coinjection with 12 pg of human or mouse *Dnmt1* protein in the majority of cases can restore the normal appearance. (*D*) The rest of the embryos have normal or even enlarged heads, but still a very short axis. (*E*) hDnmt1 protein (40 pg) coinjected with the antisense RNA results in no-axis phenotype similar to that caused by injection of 12 pg of hDnmt1 protein alone into the animal pole at two-cell stage. (*F*) Vegetal pole cells are sensitive to the same amount (12 pg) of hDnmt1 protein injected alone. Vegetal injection into two-cell blastulae leads to multiple defects as shown at stage 35.

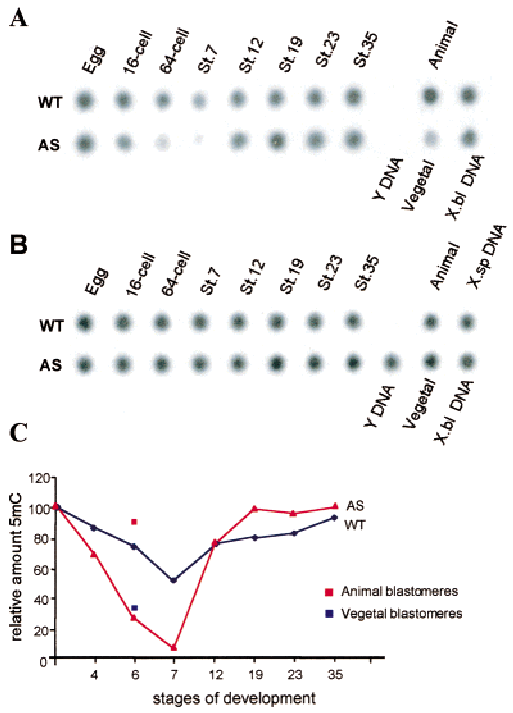

Figure 5. Changes in the levels of 5-methylcytosine (m^5C) during development of wild-type and *xDnmt1*-depleted embryos. (*A*) m^5C was detected in the DNA of wild-type (WT) and *xDnmt1*-depleted (AS) embryos at the indicated stages (from egg to stage 35 tadpole). The antibody does not recognize the nonmethylated *S. cerevisae* DNA (Y DNA). Note that there are higher levels (approximately three fold) of m^5C in the DNA that derives from the animal pole blastomeres (An) than in the DNA from the vegetal cells (Veg). (X.bl) *Xenopus* blood and (X. sp) *Xenopus* sperm DNA samples. (*B*) The blot shown in *A* was stripped from the antibody and rehybridized with a mixture of *Xenopus* and *S. cerevisae* DNA to illustrate the equal DNA loading. (*C*) The relative amount of m^5C in DNA of normal (WT) and *xDnmt1*-depleted (AS) staged *Xenopus* embryos as detected by the antibody in *A* in approximate units value. The wild-type oocyte DNA sample was taken as a standard (100%).

α-^{35}S]UTP incorporation in both normal and antisense-injected embryos (Fig. 6A,B). A higher dose of α-amanatin (20 μg/ml), which inhibits the transcription of RNA polymerase II and polymerase III, decreased [α-^{35}S]UTP incorporation to about 15–20% of that seen in the absence of the drug in both types of embryos, the remaining incorporation probably corresponds to RNA polymerase I transcription. The changes of [α-^{35}S]UTP incorporation that we observe suggests that all three types of RNA polymerases (and their associated genes) are activated in the *xDnmt1*-depleted embryos, which implies that there might be a general relaxation in chromatin structure that allows transcription to occur. In normal blastulae it is known that embryogensis is dependent on specific transcriptional cascades that are set in motion after MBT by maternally stored factors (Harland and Gerhart 1997), it is possible that this process has been disrupted by loss of DNA methylation. The net result of hypomethylation of DNA is that many genes, at least half of which are RNA polymerase- II-regulated, are not activated in their proper developmental context.

Analysis of gene expression in methylation-depleted embryos

We attempted to identify some of the transcripts that appear prematurely in the *xDnmt1*-depleted embryos by focusing on the expression level of mesoderm-specific molecules, ubiquitous and tissue-specific genes. RT-PCR analysis demonstrated that in *xDnmt1*-depleted embryos the transcripts for the secreted signaling peptide *Cerberus* (Bouwmeester et al. 1996) and the transcription factor *Xbra* (Smith et al. 1991; Bouwmeester et al. 1996) are both present at detectable levels at stage 7 (Fig. 6C). The maternally expressed transcription factor *Otx2* (Pannese et al. 1995) is also up-regulated. We could not detect any transcripts that belong to tissue-specific genes such as muscle-specific *actin* or neural β-*tubulin* (data not shown), nor could we detect any discernible change in the abundance of the heterochromatin proteins (*HP1*) α and γ in the *xDnmt1*-depleted embryos (Fig. 6C). The transcripts for linker histones *H1* and *B4* were also present in equal amounts in both control and antisense-injected embryos (data not shown). If there is a change in the expression of ubiquitously expressed genes then it is much less dramatic than that detected for the mesoderm-inducing molecules when compared to the control transcripts of *EF1*α and *H4*.

Whole-mount in situ hybridization on normal and depleted embryos at stage 7–7.5 was used to localize some of the up-regulated transcripts (Fig. 7A–C) in comparison with the nonexpressing control blastulae (Fig. 7D). In the antisense-depleted embryos, *Cerberus* transcripts are nonuniformly detected in the most anterior region of the animal pole cells (Fig. 7A). *Xbra* expression does not coincide with that of *Cerberus* but is present at the lateral dorsal area of the animal hemisphere (Fig. 7B). Neither gene was ectopically expressed in the vegetal cells (Fig. 7C). This localized pattern of both transcripts in the *xDnmt1*-depleted embryos resembles their expression during later stages of gastrulation in normal embryos (Bouwmeester et al. 1996), as they appear to be at the right place in the maternal morphogen gradient in a nonoverlapping pattern. In addition, injection of antisense *xDnmt1* RNA into a single dorsal animal cell (D1) of eight-cell blastulae allows ectopic expression of *Xbra* (Fig. 7F,G) and *Cerberus* (Fig. 7I,J) in subsets of cells that are depleted of methyltransferase (Fig. 7E- -G,I,J). The activation of *Cerberus*, *Xbra*, and *Otx2* occurs despite the fact that all of the repressive chromatin components that we analyzed are still present. Overall our analysis suggests that only genes whose promoters normally respond to maternally stored transcription factors are induced by changes in DNA methyltransferase activity.

We also found that the mesodermal markers *Cerberus*, *Xbra*, and *Otx2* are down-regulated at the equivalent of mid-gastrulation (stage 12) in the mutant embryos (Figs. 6D and 7L,M). In normal gastrula embryos there is prominent staining for *Xbra* around the blastopore,

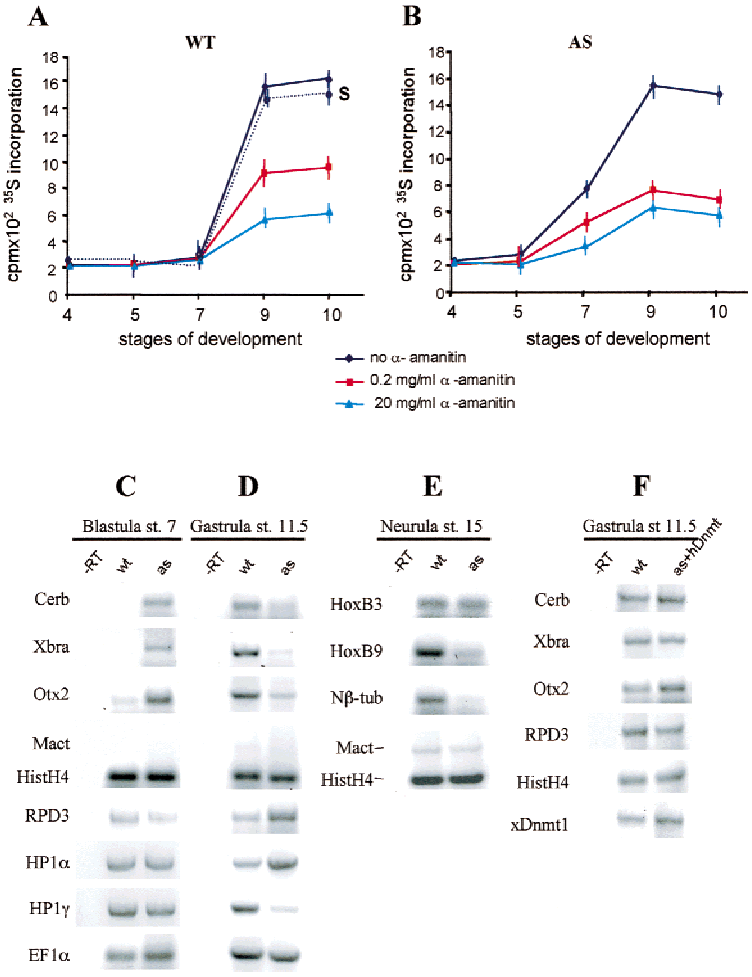

Figure 6. *xDnmt1*-depleted embryos initiate zygotic transcription before MBT. (*A*) The incorporation of microinjected (50 nCi) [α-^{35}S]UTP was used to detect the activation of gene expression in wild-type (WT) and sense *xDnmt1* RNA (S) injected embryos. Wild-type blastulae were grown also in the presense of α-amanitin for the indicated amounts. (*B*) Embryos injected with antisense RNA and 50 nCi [α-^{35}S]UTP (AS) at two-cell stage were cultured in parallel to the wild-type siblings and collected at the same time points as in *A* indicated on the graph. (*C*) RNA derived from wild-type (wt) and antisense-injected (as) embryos at stage 7 was assayed by RT-PCR for the transcripts of the mesodermal marker genes (*Cerb*, *Xbra*, *Otx2*), chromatin-related proteins (*RPD3*, *HP-1*α and γ) and ubiquitously expressed *EF1*α (*D*) RT-PCR analysis of normal (wt) and *xDnmt1*-depleted (as) embryos at stage 11.5. *Cerberus*, *Xbra*, and *Otx2* are down-regulated in the *xDnmt1*-depleted gastrulae. *RPD3* and *HP1*α are present at higher than normal levels. (*E*) RT-PCR analysis of normal (wt) and *xDnmt1*-depleted (as) embryos for the expression of the homeobox containing genes *HoxB3* and *HoxB9*, neural β-*tublin* (Nβtub), muscle-specific *actin* (Mact), and histone *H4* (Hist H4). (*F*) Antisense gastrulae rescued with 12 pg of mouse *Dnmt1* protein (as + hDnmt1) contains similar to wild-type (wt) levels of *Cerberus*, *Xbra*, *xDnmt1*, *RPD3*, and histone *H4* transcripts. The expression of *Otx2* always remained lower in the WT embryos. In *C*–*E* and *F*, –RT is a control PCR reactions of the antisense embryo RNA sample without reverse transcription.

which is considerably reduced in the *xDnmt1*-depleted siblings (Fig. 7M, cf. AS and WT). The same is observed for *Cerberus* expression in the mutant embryos (Fig. 7L). The diminished expression of essential mesodermal markers and the appearance of microcephalic and axis-truncated phenotypes in the antisense injected embryos suggests that both anterior and posterior parts of the mesoderm are affected by the deficiency of *xDnmt1* during blastula stages. This by itself can lead to the decreased expression of the posterior homeobox gene *Hox B9* and tissue-specific markers such as neural β-*tubulin* in stage 15 neurula (Fig. 6E). At the same time we saw up-regulation at gastrula of the major *Xenopus* histone deacetylase gene (*RPD 3*), *HP1*α, and, as noted earlier, the zygotic form of DNA methyltransferase (Figs 6D and 3A). The fact that most of the transcripts that show higher than normal levels belong to proteins associated with heterochromatin formation suggests that they might be regulated by a common pathway involving prematurely activated genes.

We also tested the expression of mesodermal and tissue-specific markers at mid-gastrula in the antisense-injected embryos that had been rescued by the coinjection of hDnmt1p (Fig. 6F). In all cases the expression of the analyzed genes was restored to wild-type levels with the exception of *Otx2*, which was always reproducibly higher in the rescued embryos.

The DNA methylation pattern of the Xbra *promoter in normal and antisense-depleted embryos*

We decided to test whether the premature activation of *Xbra* can be correlated with changes in the pattern of methylation of its promoter region (Artinger et al. 1997; Latinkic et al. 1997). Sequence analysis indicates that there is a considerable number of CpGs clustered around either the transcription initiation site (Fig. 8A) or an upstream region that is essential for the dose-dependent response of *Xbra* to TGFβ-activin (Latinkic et al. 1997). We analyzed the sequence from –267 to +203 using a methylation-dependent PCR assay (Singer-Sam et al. 1990) that has been modified for the use of one forward and two reverse primers (see Fig. 8A). The longer 470-bp PCR product spans three restriction sites for enzymes (1 *Hpa*II and 2 *Hha*I sites) that are inhibited by CpG methylation. When DNA is digested with *Hpa*II and *Hha*I and subjected to PCR amplification, the longer PCR product will appear only if the sites are methylated. The shorter PCR product does not traverse the restriction sites but will appear at a higher molar ratio upon digestion be-

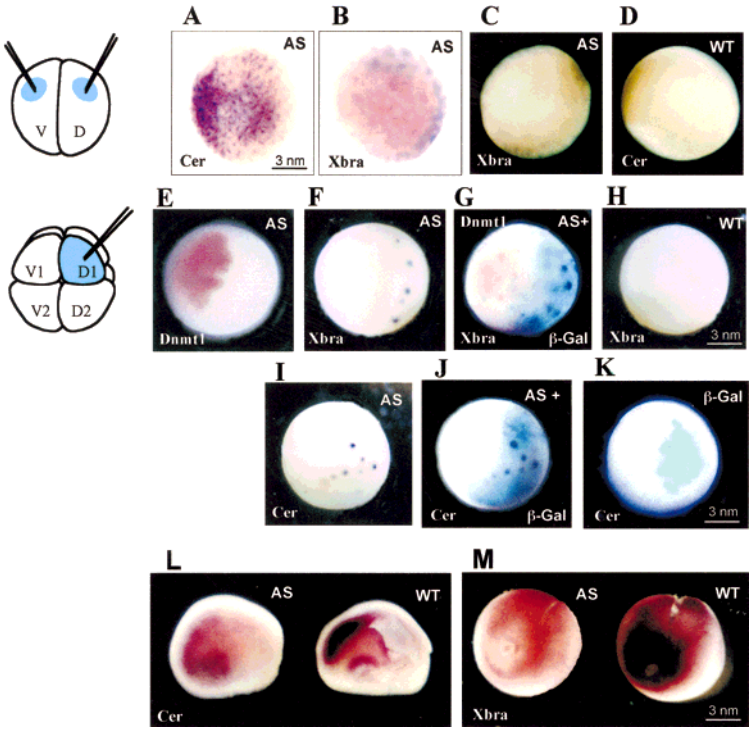

Figure 7. Localization of mesodermal markers in *xDnmt1*-depleted embryos before and after MBT. (*A*) *Cerberus* transcripts were detected by in situ hybridization in *xDnmt1*-depleted albino blastulae at stage 6.5. The albino embryos were injected symmetrically with *xDnmt1* antisense RNA (as shown on the drawing). *Cerberus* transcripts localize predominantly to the most anterior animal blastomeres (purple staining). Animal view of the embryo. (*B*) *Xbra* transcripts (purple) in the antisense blastula of the same stage (6.5) appear around the midline zone (dorsal and ventral) and never overlap with the pattern of *Cerberus* expression. Animal pole view of the blastula. (*C*) Neither of the two transcripts are aberrantly expressed in the vegetal pole blastomeres of the antisense injected embryos (hybridization with *Xbra* is shown). (*D*) Wild-type blastula at stage 6.5 do not express *Cerebrus* or *Xbra* (control hybridization with *Cerberus*). (*E*) Ectopic injection of antisense RNA and β-gal sense RNA into a single dorsal animal blastomere (D1) of eight-cell blastulae (as indicated on the drawing) depletes *xDnmt1* transcripts in a portion of animal pole cells. Only the staining for *xDnmt1* is shown (red). (*F*) Few cells of the dorsal midline zone express ectopically high levels of *Xbra* (purple). (*G*) The ectopic *Xbra* expression (dark blue) colocalizes with the staining for β-gal (blue) on the injected site of a stage 6.5 blastula. Note that *Cerberus* and *Xbra* expression localize to different populations of cells. The staining for *xDnmt1* is in red. (*H*) Uninjected embryo at stage 6.5 does not express *Xbra* or *Cerberus*. (*I*) A few of the most anterior animal cells that coincide with the site of *xDnmt1* depletion express high levels of *Cerebrus* (purple) in a stage 6.5 blastula. (*J*) The staining for *Cerebrus* transcripts colocalizes with that of the β-gal tracer. (*K*) The control embryo at the same stage (6.5) injected with β-gal RNA only do not show any staining for *Cerberus* or *Xbra* (control hybridization with Cerebrus probe). (*L*) In *xDnmt1*-depleted embryo (AS) at stage 12 the staining for *Cerberus* transcripts is reduced compared to a wild-type (WT) gastrula (*Cerebrus* in dark brown to black). The remaining transcripts still localize to the most anterior regions of dorsal mesoderm. (*M*) *Xbra* (dark brown to black staining) is dramatically reduced in *xDnmt1*-depleted gastrula at stage 12.5 (AS) as compared with the wild-type (WT) embryo.

cause of the unbalanced concentration of the reverse PCR primers (see Materials and Methods). The degree of methylation is indicated by the ratio of the 470-bp (methylated) versus 249-bp (nonmethylated) PCR products, which can be calculated from a standard curve that was generated by using a cloned *Xbra* promoter substrate with known levels of CpG methylation. (Fig. 8B,C)

Applied to normal embryos the methylation-sensitive PCR analysis indicated that the *Hpa*II and *Hha*I sites are gradually hypomethylated during gastrulation following

Figure 8. Sequences of *Xbra* promoter are hypomethylated earlier in *xDnmt1*-depleted embryos. (*A*) Schematic presentation of 1.6-kb region that covers the promoter and the first exon of *Xbra* gene. The CpG pairs in the sequence are shown by vertical lines. Underneath the CpG plot are indicated *Hpa*II and *Hha*I sites. The position of transcriptional initiation is marked by an arrow. Sequences that were analyzed by methylation-sensitive PCR during development are shown on the expanded region below. A 470-bp PCR product covers the sequences between –267 and +203 where there are two *Hha*I (Hh) sites and one *Hpa*II (H) site. The 470-bp band is produced only if the methylation-sensitive enzymes *Hpa*II and *Hha*I do not cleave in these sites. When the restriction sites are cleaved it results in the appearance of 249-bp PCR product and is indicative of hypomethylation (see Materials and Methods). Digestion with *Msp*I (which is not methylation sensitive) gives rise to the shorter product only. (*B*) To create a quantitative standard curve, a plasmid containing the *Xbra* promoter and part of the first *Xbra* exon (Latinkic et al. 1997) was methylated in vitro by *Sss*I methylase and mixed with nonmethylated *Xbra* plasmid in the combinations shown. The mixtures were digested with *Hpa*II and *Hha*I and used for PCR amplification resulting in fragments of 470 and 249, respectively. The percentage of methylation is indicated on top of each lane. M is a 100-bp DNA marker. (*C*) Standard curve generated from plotting the ratio of 470/249 fragments (X axis) against the percentage of DNA methylation (Y axis). (*D*) Genomic DNA samples from staged embryos indicated by numbers from 4–40 (stage) were digested with *Hha*I and *Hpa*II and analyzed by methylation-sensitive PCR. (*E*) Genomic DNA samples of the *xDnmt1*-depleted embryos at equivalent stages to the wild type in *B*, indicated by numbers from 4 to 35, were digested with *Hha*I and HpaII, and subjected to PCR analysis as in *B*. (*F*) The same of set of DNA samples as in *D* was digested with *Msp*I and analyzed as above. (*G*) Graphical plot of methylation changes in *Xbra* promoter during development of the normal (WT) and *xDnmt1*-depleted (AS) embryos. The ratio of 470-bp to 249-bp PCR products for each sample was quantitatively estimated and plotted against a standard curve to give the amount of methylated *Xbra* promoter as a % of the total *Xbra* promoter sequences present in the PCR reaction of genomic DNA at the indicated stages.

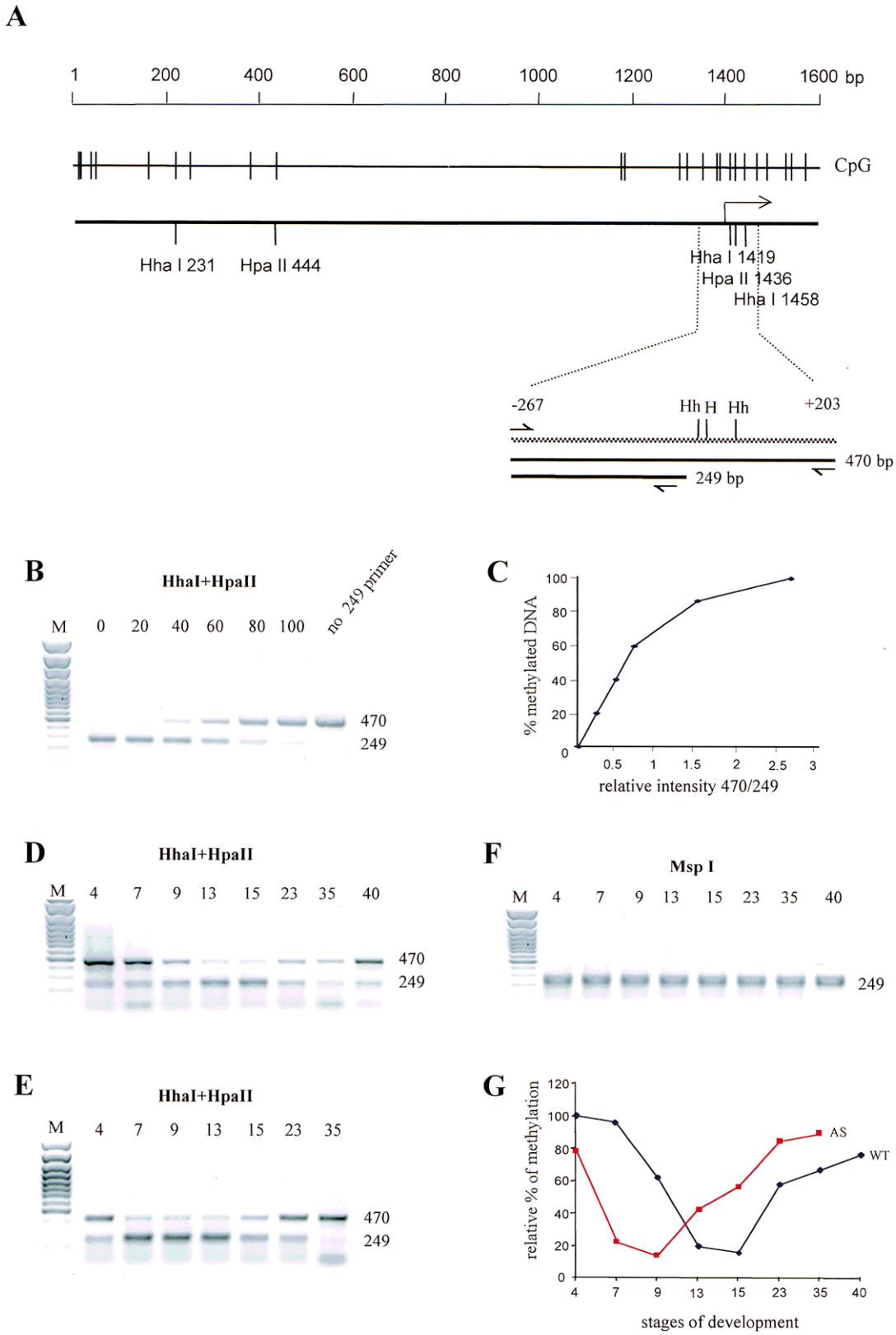

Figure 8. (*See facing page for legend.*)

Xbra expression (stage 9–13; Smith et al. 1991) and remain relatively free of methylation until stage 23 (Fig. 8D). In the *xDnmt1*-depleted embryos the pattern of methylation is different as the *Hpa*II and *Hha*I sites are already hypomethylated at stage 4 and at stage 7, which coincides with the premature activation of *Xbra* (Fig. 8E). These sites also become remethylated earlier (Fig. 8D and E, cf. Stage 15 and 23 of normal and depleted embryos, respectively). The reduced expression of *Xbra* in gastrula stage (st. 11) mutant embryos may, in part, be due to de novo methylation of promoter proximal CpGs, but this may also result from the loss of other factors that are necessary for *Xbra* expression. Although we are looking at whole-embryo DNA, the changes in methylation that we observe in normal embryos are consistent with the idea that these sites are hypomethylated as a consequence of gene activation. More copies of the promoter become hypermethylated after *Xbra* expression is restricted to a smaller population of cells in the late-stage embryo. A graph representing the methylation changes that we observe over the *Xbra* promoter is shown in Figure 8G. We obtained similar results by Southern blotting (data not shown). In brief the *Xbra* promoter undergoes developmentally programmed changes in DNA methylation that are disrupted in the *xDnmt1*-depleted embryos. The experimentally induced hypomethylation of the *Xbra* promoter correlates with its premature activation.

Discussion

xDnmt1 and DNA methylation in Xenopus *development*

In this paper we attempted to determine the temporal and spatial pattern of *xDnmt1* expression during *Xenopus* embryogenesis and found that *xDnmt1* mRNA is present at all stages of development albeit at varying levels. The transcript is very abundant in the mature oocyte and persists at high levels in cleavage stage embryos but is nonuniformly localized to the animal pole blastomeres. After MBT the maternal *xDnmt1* is replaced by a zygotic form with considerably lower expression levels. DNA derived from animal blastomeres is up to three times more methylated than DNA from the vegetal pole cells. The functional relevance of this observation is supported by the fact that vegetal blastomeres are less sensitive to injection of antisense *xDnmt1* RNA. Depletion of the maternal *xDnmt1* transcript and protein from the animal pole leads to severe developmental defects that can be largely rescued by cross-species (mouse or human) Dnmt1 proteins. Higher levels of Dnmt1 protein cannot be tolerated either by the animal or by the vegetal blastomeres. Transient depletion of maternal *xDnmt1* from *Xenopus* blastulae affects gene activation at MBT and most probably alters the ability of animal blastomeres to undergo neural induction during gastrulation and subsequent differentiation. Unlike the observation in rodents (Monk et al. 1987), but similar to zebrafish (Macleod et al. 1999), we do not detect a dramatic DNA demethylation step during the early stages of development in whole embryos. Overall m^5C content decreases toward MBT coinciding with the initiation of zygotic transcription and generally follows the decrease of *xDnmt1* levels. This suggests a passive mechanism of demethylation of *Xenopus* blastula DNA rather than the involvement of a demethylase activity.

Contribution of methylation to transcriptional silencing before MBT

The MBT is a complex event that occurs at stage 8.5 of *Xenopus* development and is associated with major changes in the cell cycle, the appearance of cell cycle checkpoints and initiation of zygotic transcription (Newport and Kirschner 1982a; Howe and Newport 1996). A number of studies have demonstrated that in the early embryo, chromatin assembly efficiently competes with binding of transcription factors to the promoter elements (Prioleau et al. 1994; Almouzni and Wolffe 1995). This competition can be experimentally shifted in favor of transcription either by prebinding of transcription factors to microinjected reporter templates (Prioleau et al. 1994; Almouzni and Wolffe 1995) or by treatment of cultured blastomeres with a high concentration of TGF (Kinoshita et al. 1993). Toward MBT the decrease in histone concentration and the coupling of their synthesis to the cell cycle allows transcription to be initiated at a specific nucleus to cytoplasm ratio (Newport and Kirschner 1982b). Somatic linker histones have been shown to play a role in differentiated gene expression (Bouvet et al. 1994; Kandolf 1994) and in the duration of mesodermal competence during gastrulation (Steinbach et al. 1997) whereas the contribution of maternal linker histone, H1M, to overall transcriptional silencing before MBT is unknown.

Recent studies (Jones et al. 1998) and our own observations (R. Meehan and I. Stancheva, unpubl.) have found that *Xenopus* oocytes and early embryos contain a considerable amount of the methylation specific transcriptional repressor protein MeCP2. This methyl–CpG binding protein acts as a global transcriptional repressor in two ways, either directly inhibiting binding of transcription factors to methylated promoters (Nan et al. 1997) and at the level of chromatin via interaction with Sin3A/histone deacetylase complex (Jones et al. 1998; Nan et al. 1998). Mutation of the *MeCP2* gene also causes embryonic lethality in mice but there are no reports that there is a failure of X-inactivation or inappropriate expression of imprinted genes in these mutants (Tate et al. 1996). It is probable that *MeCP2* in mice and *Xenopus* is performing a similar function.

In our experiments transient depletion of maternal *xDnmt1* RNA and protein by antisense RNA injection dramatically decreases the content of m^5C during the rapid early cleavages. Presumably hypomethylation leads to the exclusion of MeCP2 from chromatin and allows premature gene activation. Interestingly, as suggested by [α-^{35}S] UTP incorporation in the presence of α-amanitin, *xDnmt1*-depleted blastulae can activate the

genes transcribed by all three RNA polymerases approximately two cell cycles before MBT (stage 6.5). Our limited RT-PCR analysis allowed us to identify upregulated mRNAs for the mesoderm inducing molecules *Cerberus*, *Xbra*, and *Otx2*, however none of these genes can be characterized as being tissue specific in their expression patterns at this stage of development (Smith et al. 1991; Pannese et al. 1995; Bouwmeester et al. 1996). The symmetrical injection of antisense *xDnmt1* RNA into both blastomeres at two-cell stage and the ectopic injection into a single animal dorsal blastomere (D1) showed that *Cerberus* and *Xbra* transcripts appear in nonoverlapping population of cells and that their patterns of expression greatly resemble that of normal gastrulae (Smith et al. 1991; Bouwmeester et al. 1996). This is consistent with the idea that the *Xenopus* embryo is prepatterned by maternal gradients of transcription factors and morphogens before MBT (Heasman 1997). Additional experiments are required to identify the full spectrum of genes that are activated in *xDnmt1*-depleted *Xenopus* blastulae.

The phenotypes of *xDnmt1*-depleted embryos during gastrulation and at the equivalent of stage 35 suggest that loss of methylation during cleavage stages cannot be completely compensated by even higher than normal levels of zygotic methyltransferase. In addition the mesodermal markers *Cerberus*, *Xbra*, and *Otx2* are downregulated during gastrulation, when normally they are highly expressed (Smith et al, 1991; Pannese et al, 1995; Bouwmeester et al, 1996). One possibility is that loss of methylation and premature expression of genes causes inappropriate timing of mesoderm induction and leads to negative interference of signalling pathways. On the other hand, we do not know how essential is the loss *xDnmt1* and respectively of the initial methylation patterns for the ability of animal pole blastomeres to differentiate since this potential must also be set up before MBT (Kinoshita et al. 1993). Disturbed gastrulation movements of antisense-injected embryos suggests that they face serious signaling problems. Despite this fact, they seem to divide in an indistinguishable fashion compared with wild-type siblings before gastrulation (R. Meehan and I. Stancheva, unpubl.). It also cannot be excluded that premature gene activation may affect the cell cycle and lead to the appearance earlier than usual of cell cycle checkpoints, which results in untimely apoptosis and cell death.

Does DNA methylation have a conserved role in regulation of developmental gene activation?

At present it has been demonstrated that mice, zebrafish, and frog contain abundant oocyte forms of *Dnmt1*. Loss or inhibition of the enzyme during early stages is either lethal or leads to abnormal development (Li et al. 1992; Lei et al. 1996; Martin et al. 1999). The maternal forms of *Dnmt1* in zebrafish and *Xenopus* are both present at high levels in the animal pole in oocytes, eggs, and animal blastomeres during early cleavages (Martin et al. 1999; this study). The polarized localization reflects the general uneven distribution of maternal cytoplasm factors that is essential for mesoderm induction both in *Xenopus* and zebrafish (Wolpert et al. 1998). Oocyte *xDnmt1* is almost equally distributed in the nucleus and cytoplasm (Kimura et al. 1999) and RNA transcript levels drop dramatically after the breakdown of germinal vesicle (R. Meehan and I. Stancheva, unpubl.; see also Fig. 1). In mice there is no compartmentalization of the oocyte cytoplasm and the adjustment of nuclear levels of *Dnmt1* enzyme is achieved by migration of the protein during oocyte maturation (Carlson et al. 1992; Mertineit et al. 1998). In the course of embryo development in all species *Dnmt1* transcripts were found at persistently high levels in the cells of neural origin, an observation that still remains functionally unclear (Goto et al. 1994; Martin et al. 1999). It has been argued that in mice DNA methyltransferase is not essential for the early embryonic cells. Mouse ES $Dnmt^{-/-}$ cells are viable in culture but die upon differentiation (Lei et al. 1996). Somatic DNA methylation patterns in mouse embryos are established after a wave of genome-wide demethylation during preimplantation (Monk et al. 1987; Razin and Kafri 1994; Panning and Jaenisch 1996). Such dramatic changes in m^5C levels were not detected at the onset of gastrulation in *Xenopus* and zebrafish (Macleod et al. 1999; this study). The asymmetric localization of components derived from the maternal cytoplasm and *Dnmt1* itself in these species may allow differential methylation patterns to be set up in subsets of cells during the cleavage stages. These patterns will be modified and reinforced by the localized differential expression of the zygotic *Dnmt1* during later stages of development. Despite the considerable differences that exist between mammalian development and that of amphibia and fish, it is clear that reduced levels of *Dnmt1* and DNA hypomethylation at the onset of embryogenesis result in equally severe phenotypes that bear distinguishable similarities between the different species as indicated by the presence of axial defects, failure to form neural tissue, and improper patterning of the somites (Trasler et al. 1996; Martin et al. 1999). In *Xenopus* and zebrafish, mesoderm formation is negatively affected during gastrulation by hypomethylation and some genes such as *Cerberus*, *Otx2*, *Xbra*, and the zebrafish *floating head* and the brachyury homolog *no tail* (Martin et al. 1999) are expressed at reduced levels. We show here that maternal *xDnmt1* in *Xenopus* is essential for maintenance of gene silencing before midblastula transition and that changes in *xDnmt1* levels affect differentiation of animal pole blastomeres. Our studies of the *Xbra* promoter revealed that it undergoes developmentally regulated changes of DNA methylation that coincide with the timing of *Xbra* expression. Bird (1992) has produced elegant models for transcriptional repression by DNA methylation that depend on a number of parameters: (1) the strength of the promoter; (2) the number and location of methylated CpG pairs; and (3) the presence of methyl-binding proteins that can interact with heterochromatin promoting factors. In this dynamic view of methylation-mediated gene inactivation there is a shifting balance between activation and repression, which depends on

the presence of strong transcription factors to activate a methylated gene. Such a scenario may be the normal mode of gene activation during development. Unfortunately this question has not been answered in mice because of the complexity of $Dnmt^{-/-}$ phenotypes, which is greatly dominated by the negative effect of misexpression of imprinted genes and X-chromosome inactivation (Panning and Jaenisch 1996). We also find that hypomethylation of *Xenopus* blastulae DNA does not lead to aberrant expression of tissue specific genes, which supports similar observations in mice (Walsh and Bestor 1999). It is possible that many more aspects of the methylation repression machinery are conserved between mammals and amphibia.

Materials and methods

Cloning of xDnmt1 *cDNAs*

A stage 20–22 *Xenopus* λ ZapII cDNA library was screened with a pair of [γ-^{32}P]dATP end-labeled oligonucleotides corresponding to the carboxy-terminal *xDnmt* sequences: 3811–3831, TTCCAGAGGCAGATTCGTGG and 4271–4291, ACACTCACCACACGATGC. The longest *xDnmt1* clone (GenBank accession no.: AF192996) p9/19 containing a 1.4-kb insert in pBluescript SK+ was sequenced and used as a hybridization probe for Northern blot analysis and for synthesis of sense and antisense RNA. A partial 830-bp cDNA *xDnmt1* p59/889 corresponding to the oocyte *xDnmt1* (Kimura et al. 1996) amino-terminal sequences between base pairs 59 and 889 was obtained by RT-PCR amplification (TrueSprinter RT–PCR kit, Hybaid Ltd.) from *Xenopus* oocyte RNA using the following primers: (f) ACTGTGTCCTGTTGATTCGC, (r) TTCTTCCGCATCAGACCG. The RT-PCR product was cloned into *Stu*I site of pCS2+ plasmid.

Embryos and microinjections

Xenopus embryos were obtained from in vitro-fertilized wild-type and albino eggs, grown and microinjected according to the standard procedures. Staging was according to Nieuwkoop and Faber (1967). Blastulae (2-, 4-, 8-, and 16-cell) were injected with 120, 400, 520, and 600 pg of antisense RNA per cell or 520 pg of sense capped RNA synthesized in vitro from xDnmt p9/19 (T3/T7 Cap-Scribe kit, Boehringer) RNA was injected into the amimal or vegetal half of the embryos as far as possible from the midline. The ectopic injections of 250 pg of sense or antisense *xDnmt1* RNA coinjected with 100 pg of cytoplasmic β-gal sense RNA were performed into the D1 blastomere of albino eight-cell stage embryos (n = 150). The hDnmt1 and mDnmt1 baculovirus-produced proteins (Pradhan et al. 1997) were diluted in sterile water to a final concentration of 1, 3, and 8 ng/μl or in a sterile water containing 130 ng/μl (final concentration) antisense *xDnmt1* RNA prior to microinjection.

Whole-mount in situ hybridization

The whole-mount in situ hybridizations were performed as described (Harland 1991). Digoxigenin-UTP or fluorescein-labeled probes were prepared using Boehringer reagents. *Xbra* probe derived from psp73 plasmid (Smith et al. 1991), *Cerberus* probe was synthesized as described (Bouwmeester et al. 1996), and *xDnmt1* 3′ and 5′ probes were made from *xDnmt1* p9/19 and *xDnmt1* p59/889, respectively. β-Gal staining was performed as described previously (Steinbach et al. 1997).

Northern blot analysis

RNA isolation and Northern blots were carried out according to the standard procedures.

RNA (15 μg) from each stage were loaded on the gels. The blots were hybridized with 1.4-kb *xDnmt1* cDNA [α-^{32}P]dCTP-labeled probe after stripping the filters with *Xenopus* ODC probe (Isaacs et al. 1992). The signals were detected by conventional autoradiography and by FLA 2000 FluoroImager (Fuji-Film) and IP reading, quantified by Aida 2.0 (Advanced Image Digital Analyser) software (FujiFilm, Ltd.), and plotted using Microsoft Excel.

Whole embryo run-on experiments

Pigmented two-cell-stage embryos were coinjected with 520 pg of antisense or sense *xDnmt1* RNA and 50 nCi [α-^{35}S]UTP (400 Ci/mmole, Amersham) and cultured in 4% Ficoll, 0.2× Marc's Modified Ringers or in the same buffer containing either 0.2 μg/ml or 20 μg/ml α-amanitin in parallel with the control siblings injected with 50 nCi [α-^{35}S]UTP only. Starting from stage 4, samples of 15 injected and control embryos were collected at each time point and frozen at –20°C prior to being processed. Total RNA was purified by the use of PureScript RNA isolation kit (Gentra) and 3 aliquots of 10 μl from each sample (final volume of 50 μl) were immobilized on glass fiber filters (GF/A) (Whatman). The filters were washed subsequently with ice-cold 20%, 10%, and 5% trichloracetic acid. Incorporated label was detected by a Liquid Scintilation Analyzer 1900CA (Packard).

RT–PCR analysis

Total RNA (1 μg) from normal and *xDnmt1*-depleted embryos was treated with RNase free DNase RQ1 (Promega) and reverse transcribed with SuperScript II (BRL Life Technologies). cDNAs were subjected to serial dilutions and with control primers for *EF1α* (Wilson and Melton 1994) or *Histone H4* (f:GGGATAACATTCAGGGTATC, r:CATGGCGGTAACTGTCTTC) to estimate the linear range of PCR amplification. The other pairs of RT-PCR primers were:

xDnmt1 amino-terminal (f: TCTTGTGGATGAATGCGAGG, r:CCACATCATCCTTCCTCT),

xDnmt1 sense/antisense overlap (f:GGCGGTGCAAGGACATTG, r:ACTGGTAGCCCATGCGTAC),

HP1α (f: CTCAGAGGAGCATAACACTTGG, r:CCTTCTTCATTCAGACACACA),

HP1γ (f:CAAGAAGGTGGAGGAAGC, r:CCAGAGGATGAAGCACAATAAA),

RPD3 (f:ACGGTGATGGTGTTGAGG; r:AGCAACGAGCCACATTCC),

Xbra (f:TGGCTTATTCCTAATGGTGG, r:CTGGCTGTGACTCATTGG),

Cerberus (f:TCATAAGAGCAACTTCCACC, r:TGCTGATTGGTTGTTAGTCC),

Otx2 (f:TACCTGAGTCCAGAGTCC, r:CTGCTGGTAGGTCATAGG),

HoxB9 (f:TACTTACGGGCTTGGCTGGA, r:AGCGTGTAACCAGTTGGCTG),

HoxB3 (f:ATATGATGAGCCACGCAGCAG, r: CAGATGCTGCAGCTCTTTGGC),

Neural β-tublin (f: ACACGGCATTGATCCTACAG, r: AGCTCCTTCGGTGTAATGAC),

muscle *actin* (f: GCTGACAGAATGCAGAAG, r: TTGCTTGGAGGAGTGTGT).

Amplification was performed for *Xbra*, *Otx2*, *Cerberus*, *HoxB9*, and *RPD3* (35 cycles) and for *Histone H4*, *HP1*α and γ, *EF1*α (28 cycles). PCR reactions were analyzed in 1.2% agarose gels and directly scanned by FLA 2000 FluoroImmager (FujiFilm). All RT-PCRs were repeated at least three times for every set of primers and RNAs and were also peformed in the absence of reverse transcriptase (–RT).

Immunological detection of m^5C

DNA was isolated from 25–50 staged wild-type, antisense *xDnmt1* RNA-injected embryos or from ~250 stage 6 wild-type blastulae that had been separated into animal and vegetal halves. DNA (5 µg) from each sample was digested with *Eco*RI and transferred to Z-probe membrane (BioRad). Dot blots were probed for m^5C as described (Reynaud et al. 1992; Tweedie et al. 1997) using a monoclonal m^5C antibody and a secondary anti-mouse HRP conjugated IgG. Chemiluminiscence signal (ECL reagents, Amersham, Life Technologies) was detected by exposure to Fuji XR films and by scanning with FLA 2000.

Methylation-sensitive PCR analysis

PCR quantification of methylation at the *Xbra* promoter was based on a method described by Singer-Sam et al. (1990) and modified for the use of three primers. Total genomic DNA from staged normal and *xDnmt1*-depleted embryos was digested to completion with *Hpa*II and *Hha*I restriction enzymes or with *Msp*I. The complete digestion was monitored by removing a portion of the reaction at time zero and incubating it with a control plasmid (pBluescript SK+). After digestion the samples were phenol-chloroform, chloroform extracted, ethanol precipitated, and resuspended in distilled water to a final concentration of 100 ng/µl. Undigested DNA samples were tested for linear amplification range with both pair of primers (see below) as a measure of equal template concentration and by the combination of all three primers that gave linear amplification. Competitive PCR reactions (100 µl) typically included 2 µl of *Hpa*II, *Hha*I, or *Msp*I-digested genomic DNA (20 ng), 7 pmoles of forward primer, 5 pmoles of first reverse primer (249), and 7 pmoles of second reverse primer (470). The following amplification cycles were used: denaturing at 95°C for 30 sec, annealing for 30 sec at 51°C, 1-min elongation at 72°C, 35 cycles. *Xbra* promoter primers were as follows: forward CAATCAGCAGTTGCCTCAC; 1st reverse CTTCGTAACACACAGACTGG; 2nd reverse ACCTTCCATTCTTAGTGACG. To create a quantitative standard curve a plasmid containing the *Xbra* promoter and part of the first *Xbra* exon (Latinkic et al. 1997) was methylated in vitro by *Sss*I methylase (2 hr with addition of SAM after the first hour of incubation). Methylated plasmid (m*Xbra*) was mixed with unmethylated *Xbra* plasmid in combinations (vol/vol) 10:0, 8:2, 6:4, 4:6, 2:8, and 0:10-, all of the mixtures were digested either with *Hpa*II and *Hha*I or with *Msp*I, and 10 ng of each mixture were used for PCR amplification as described above. All reactions were ethanol precipitated, dissolved in 15 µl of 1× loading buffer, and electrophoresed in 1.2% agarose gel containing 0.5 µg/ml ethidium bromide. The gels were scanned by FLA 2000 Fluoroimmager (475 nm excitation index) and the intensity of both 249-bp and 470-bp PCR products was determined by Aida 2.0 software as a peak function in approximate units (AU). The ratio of 470-bp to 249-bp PCR products were plotted versus % of methylation (% m*Xbra* in the reaction) for each mixture sample. The standard curve was used to quantify the relative % of methylation for the endogenous genomic *Xbra* promoter sequences by plotting 470/249 values from the agarose gels scans of each reaction for wild type and *xDnmt1*-depleted embryos against the standard. Each series of PCRs was repeated at least 3 times.

Protein extracts and immunoblots

Total protein extracts were prepared from staged wild-type eggs, wild-type and antisense *xDnmt1*-depleted blastulae and gastrulae as described (Evans and Kay 1991) run in 7% SDS-polyacrylamide gels and electrotransfered to PVDF membrane. *xDnm1* was detected by a polyclonal antibody raised against the conserved carboxy-terminal domain of mouse *Dnmt1* (Liu et al. 1998), PCNA was detected by a monoclonal PC 10 antibody (Waseem and Lane 1990). Anti-mouse or anti-rabbit HRP-conjugated IgGs were used as secondary antibodies. For the immunoprecipitation experiments, protein extracts were prepared from ~200 wild-type 64-cell blastulae, which had been dissected to animal and vegetal halves. The immunoprecipitation was performed according to the standard procedures. Sepharose-proteinA beads were washed extensively with buffer containing 50 mM Tris, at pH 7.5, 150 mM NaCl, 1% NP-40, and 0.5% Na deoxocholate. The bound fractions were extracted and run in 7% SDS-polyacrylamide gels. *xDnmt1* protein was detected by mouse monoclonal antibody against human *Dnmt1*.

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